DAIDS

VIROLOGY MANUAL

FOR HIV LABORATORIES

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Compiled by

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and

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CYTOMEGALOVIRUS DRUG SUSCEPTIBILITY ASSAY
PLAQUE REDUCTION METHOD

I. PRINCIPLE

Cytomegalovirus (CMV) infections are a major cause of morbidity and mortality among immunocompromised patients. Ganciclovir (DHPG) and Foscarnet (PFA) are antiviral compounds with excellent in vitro activity against CMV. Their use in clinical trials have shown some benefit in treatment of severe CMV infections. The majority of clinical strains of CMV are inhibited by Ganciclovir, showing IC_{50} values below approximately 6 µM, and by Foscarnet, showing IC_{50} values below approximately 400 µM. However, there are recent reports of patients showing progressive CMV disease caused by drug resistant strains of CMV. Whether CMV strains resistant to Ganciclovir and/or Foscarnet will become a major clinical problem as the use of these drugs become more extensive is not known. Therefore, continuous surveillance of sensitivities of CMV isolates to antivirals has become crucial.

The CMV sensitivity assay requires host cells, usually human foreskin fibroblasts (MRHF), infected with the patients virus. The patients CMV strain is tested in parallel with a control strain such as AD-169. The viruses are inoculated into wells containing MRHF cells; after infection the number of plaque forming colonies (PFC) is determined. A predetermined number of PFC are inoculated into the wells of a 24-well plate. After adsorption, the wells are overlaid with agarose containing various concentrations of drug and incubated. When there are an adequate number of plaques in the control wells (wells without drug), the plaques in all wells are stained and counted. The number of PFC in the wells containing drug are compared to those in the control wells and an IC_{50} is calculated.

II. SPECIMEN REQUIREMENTS

CMV isolates, fresh or frozen in LN_2, grown to demonstrate 50-100% CPE.

III. REAGENTS

Trypsin-EDTA--Store at 4-8^{0}C. Observe manufacturer's outdate.

Human Foreskin fibroblasts MRHF (Biowhitaker catalog number 72-213B for 75 mm^2 flask or 72-213F for ampoules of cryopreserved cells). (Procedure for cryopreserved cells can be obtained from Biowhittaker catalog).

Minimum essential medium (Eagle)(MEME)--Store at 4-8^{0}C. Observe manufacturer's outdate.

Fetal Bovine Serum (FBS)--Store frozen at -20^{0}C. Observe manufacturer's outdate. When needed, rapidly thaw a bottle in a 37^{0}C waterbath, then heat-inactivate in a 56^{0}C waterbath for 30 minutes with occasional shaking. The level of water in the waterbath should be as high as the

level of the serum in the bottle. Store at 4-8^{0}C after thawing. Heat-inactivated FBS has a one month outdate.

Phosphate Buffered Saline (PBS) with calcium or magnesium or Hanks Balanced Salt Solution (HBSS)--Store at room temperature. Observe manufacturer's outdate or a one month outdate after opening.

SeaPlaque Agarose--may be obtained from FMC Bioproducts, Rockland, ME). Store at room temperature. Observe manufacturer's outdate.

Ganciclovir (DHPG)--Provided by the Virology Quality Assurance Laboratory (VQA). Store at -70^{0}C or below.

Foscarnet (PFA)--Provided by the VQA. Store at -70^{0}C or below.

Formalin--Store at room temperature. Observe manufacturer's outdate.

Dimethylsulfoxide (DMSO)--Store at room temperature. Observe manufacturer's outdate.

Reagent grade water.

AD169 CMV (clinical cell-associated sensitive strain)--Store in LN_2.

Resistant CMV strain--Store in LN_2.

Ethanol--Store at room temperature. Observe manufacturer's outdate.

Crystal Violet--Store at room temperature. Observe manufacturer's outdate. Prepare 0.8% in 50% ethanol.

Growth Medium (10%). To make 500 mL:

a) 450 mL MEME
b) 50 mL FBS

Growth Medium (2%). To make 500 mL:

a) 490 mL MEME
b) 10 mL FBS

Cryoprotective Medium. To make 10 mL:

a) 7 mL MEME
b) 2 mL FBS
c) 1 mL DMSO

Thaw Medium. To make 10 mL:

a) 9 mL MEME
b) 1 mL FBS

Agarose

a) To prepare a 2X stock (0.8%), weigh 4.0 gm agarose.
b) Add reagent grade water to 500 mL.
c) Autoclave at 15 lbs for 15 minutes at 121^0C.
d) Keep in 56^0C waterbath until used. If prepared in advance, store at 4^0C; boil stock prior to use; let cool in 56^0C waterbath.
e) When ready to overlay plates after virus adsorption, mix 2X concentrations of drug in 2X MEME with 10% FBS and antibiotics with equal volumes of 2X (0.8%) agarose. Final concentration of overlay: 0.4% agarose in MEME with 5% FBS.

Note: Keep MEME at 4^0C until used. Be sure overlay is not too hot before proceeding.

Ganciclovir Concentrations

a) The following <u>final</u> (1X) drug concentrations will be used in the assay:
GCV (µM):

8 concentrations (2-fold dilutions)	0	1.5	3	6	12	24	48	96

b) The drug concentrations are prepared from stock solutions provided by the VQA Laboratory and kept at -70^0C until used. At the time of overlay preparation, thaw drug and warm in water bath, shaking intermittently and/or vortexing or sonicating in a waterbath to <u>ensure complete dissolution</u>.

c) Prepare 2X concentrations of drugs in 2X MEME with 10% FBS and antibiotics. In the final step, add to an equal volume of 0.8% agarose to give final concentration.

d) To overlay 1.5 mL per well in a 24-well plate, 3 wells per drug concentration, prepare 5 mL of each drug concentration in final overlay for each plate inoculated to ensure adequate volume.

<u>For actual volumes used, multiply the volumes given in the table by the number of viruses assayed.</u>

Remember, drug-sensitive and drug-resistant controls must be included in each assay. Therefore at least 3 plates (2 controls, 1 unknown) would be prepared for each assay.

Calculations are based on ganciclovir (GCV) stock concentration of 4.5 mM and serial 2-fold dilutions of drug.

Be sure stock drug is in solution before proceeding.

2X Ganciclovir Concentrations

Initial drug conc	**4.5 mM**	**384 µM**	**192 µM**	**96 µM**	**48 µM**	**24 µM**	**12 µM**	**6 µM**	**0 µM**
Add drug above (volume)	300 µL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	
to 2x MEME/ 10% FBS	3216 µL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	
Yields(2X) GCV conc	**384 µM**	**192 µM**	**96 µM**	**48 µM**	**24 µM**	**12 µM**	**6 µM**	**3 µM**	
Take (mL) to prepare next dilution	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	N.A.	
Volume remaining	N.A.	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	6 mL	

Final Ganciclovir Concentrations in Agarose Overlay (using 2X from above)

GCV (2X)	**N.A.**	**192 µM**	**96 µM**	**48 µM**	**24 µM**	**12 µM**	**6 µM**	**3 µM**	**0 µM**
Take 2X GCV (mL)		2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL
Add 0.8% agarose (mL)		2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL
Yields final (1X) GCV		**96 µM**	**48 µM**	**24 µM**	**12 µM**	**6 µM**	**3 µM**	**1.5µM**	**0 µM**
Overlay (1X) 0.4% agarose/ MEME 5% FBS (mL)		5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL

Foscarnet Concentrations

a) The following final (1X) drug concentrations will be used in the assay:
PFA (µM):
8 concentrations 0 25 50 100 200 400 800 1000
(2-fold dilutions except for 1000µM)

b) The drug concentrations are prepared from stock solutions provided by VQA Laboratory and kept at -70^{0}C until used. At the time of overlay preparation, thaw drug and warm in water bath, shaking intermittently and/or vortexing or sonicating in a waterbath to ensure complete dissolution.

c) Prepare 2X concentrations of drugs in 2X MEME with 10% FBS and antibiotics. In the final step, add to an equal volume of 0.8% agarose to give final concentration.

d) To overlay 1.5 mL per well in a 24-well plate, 3 wells per drug concentration, prepare 5 mL of each drug concentration in final overlay for each plate inoculated to ensure adequate volume. For actual volumes used, multiply the volumes given in the table by the number of viruses assayed.

Remember, drug-sensitive and drug-resistant controls must be included in each assay. Therefore at least 3 plates (2 controls, 1 unknown) would be prepared for each assay.

Calculations are based on Foscarnet (PFA) stock concentration of 20 mM and serial 2-fold dilutions except for the initial 1000 µM concentration.

Be sure stock drug is in solution before proceeding.

2X PFA Concentrations

Initial drug conc	**20 mM**	**2000 µM**	**1600 µM**	**800 µM**	**400 µM**	**200 µM**	**100 µM**	**0 µM**
Add drug above (volume)	0.8 mL	4.8 mL	3 mL	3 mL	3 mL	3 mL	3 mL	
to 2x MEME/ 10%FBS	7.2 mL	1.2 mL	3 mL	3 mL	3 mL	3 mL	3 mL	
Yields(2X) PFA conc	**2000 µM**	**1600 µM**	**800 µM**	**400 µM**	**200 µM**	**100 µM**	**50 µM**	
Take (mL) to prepare next dilution	4.8 mL	3 mL	3 mL	3 mL	3 mL	3 mL	N.A.	
Volume remaining	3.2 mL	3 mL	3 mL	3 mL	3 mL	3 mL	6 mL	

Final PFA Concentrations in Agarose (using 2X from above)

PFA (2X)	**2000 µM**	**1600 µM**	**800 µM**	**400 µM**	**200 µM**	**100 µM**	**50 µM**	**0 µM**
Take 2X drug (mL)	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL
Add 0.8% agarose (mL)	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL
Yields final (1X) PFA	**1000 µM**	**800 µM**	**400 µM**	**200 µM**	**100 µM**	**50 µM**	**25 µM**	**0 µM**
Overlay (1X) 0.4% agarose/ MEME 5% FBS (mL)	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL

IV. EQUIPMENT AND SUPPLIES

Laminar flow (Class 2 biosafety hood)
Gloves
Lab coat
Sterile disposable 1 mL, 2 mL, 5 mL, and 10 mL plugged and unplugged Pasteur pipettes
Sterile 15 mL and 50 mL tubes
25 cm^2 tissue culture flask
24-well tissue culture plates
Sterile cryovials
Hemacytometer
Microscope (inverted, dissecting or phase with low power)
Vacuum aspirator with appropriate trap
Waterbath capable of 56^0C
Incubator at 36-37^0C with 5% CO_2 and humidity
Low speed centrifuge with tube and plate carriers

V. PROCEDURE

A. Propagation and assay of cell-associated virus

1. Obtain CMV clinical isolate, usually a culture in a screw cap tube.

2. Trypsinize cells and transfer cell suspension to a 25 cm^2 flask of human fibroblast cells. When cytopathic effect (CPE) is observed, trypsinize and redistribute cells to provide a more rapid progression of CPE.

3. Pipette medium from the flasks.

4. Rinse monolayer twice with PBS without calcium or magnesium. Add 1-3 mL fresh trypsin to flask. Place at room temperature or at 37^0C for approximately 1-2 minutes or the minimum time needed to loosen the cells.

5. Tap flask sharply to dislodge cell monolayer.

6. Add 5 mL of growth medium, pipette up and down to separate cell clumps, then transfer contents to a fresh 25 cm^2 flask. In some cases, it may be necessary to add fresh fibroblast cells as well.

7. Observe the flask daily and repeat every 3-4 days until the CPE reaches at least 50%.

8. When CPE involves 50-75% of the monolayer, trypsinize cells, gently resuspend in MEME containing 10% FBS and antibiotics; pipette up and down to obtain a single cell suspension.

9. Count cells in a hemacytometer.

Note: Accurate results depend upon having a uniform distribution of a single-cell suspension.

10. Multiply cell count by the estimated percent of viable infected cells to determine the number of plaque-forming cells (PFC) in the suspension.

11. Adjust cell concentration to 400 PFC/mL to provide an inoculum dose of 60-80 PFC/0.2 mL.

12. Prepare in 10% MEME approximately 6 mL of virus stock per panel to be inoculated. Inoculation of panels on the same day is recommended. (Note: 8-10% MEME is used for cell-associated virus stocks and 2% MEME for cell-free virus stocks.)

13. Freeze aliquots of remaining virus stock for retest if needed.

    a. Centrifuge the infected cell suspension at low speed so as not to pack the cells too tightly (i.e., 120-180 x g for 5-10 min).

    b. Resuspend the cell pellet in cryoprotective medium; keep well-mixed since clumping will result in variability in titer between aliquots.

    c. Freeze at a titer of 4×10^5 PFC/mL.

    d. Aliquot into small cryovials (0.5 mL/vial).

e. Freeze slowly and store at -85^{0}C or colder.

f. Thaw quickly prior to use; dilute in MEME with 10% FBS to obtain 400 PFC/mL.

(Note: Freezing of virus stock before assay may contribute in variability in the inoculum dose.)

B. Preparation of MRHF 24-well plates from flasks

Note: The time to dislodge cells from a flask will vary with age of the cells and the activity of the trypsin. The goal is to minimize cell contact with trypsin and to grow healthy cells.

1. Obtain MRHF cells at a population doubling level (PDL) of 23-24. Cells can be used up to PDL 30-35 . Records should be maintained of PDL and cultures labeled accordingly. (To calculate PDL: For cells obtained at PDL 24, after passage at a 1:2 split, the cells would be PDL 25. After passage at a 1:4 split, the cells would be PDL 26.)

2. Aspirate medium from 75 cm^2 flask.

3. Rinse monolayer twice with HBSS or PBS (without calcium or magnesium).

4. Add 3 mL of trypsin to flask.

5. Incubate at room temperature or at 37^{0}C for 1-3 min or the minimum time to loosen the cells.

6. Examine monolayer under the microscope and when cells are rounded, rap flask to dislodge cells.

7. Add 10 mL of 10% MEME and pipette up and down to break up clumps.

8. Add 10% MEME to 75 mL total volume. Pipette to mix well.

9. Seed three 24-well plates with 1.0 mL of cell suspension per well, a 1:2 split.

10. Incubate at 37^{0}C in CO_2 incubator until confluent.

11. Inoculate panels when cells are just confluent, usually 2 days after seeding at a 1:2 split.

12. For each virus isolate, prepare one 24 well panel of MRHF for each drug tested. Note: Subconfluent cells are more sensitive, but may be more fragile. At higher

passage levels, cells may require a longer period to become confluent and are more likely to peel.

C. Plaque reduction assay for drug susceptibility screening of CMV

1. For each virus tested, use one 24-well panel per drug assayed. When assaying cell-associated viruses, do not aspirate the medium from the wells prior to inoculation.

2. Gently pipette virus stock up and down to ensure well-mixed, single cell suspension, then inoculate each well with 0.2 mL of cell suspension containing 60-80 PFC of cell-associated virus.

3. Allow adsorption for 90 min in a 37^0C CO_2 incubator.

4. During the adsorption step, prepare drug concentrations in overlay solutions.

5. After adsorption, carefully aspirate inoculum and medium from the wells. Overlay wells with 1.5 mL 0.4% agarose containing the appropriate concentration of antiviral, 3 wells per drug concentration. Allow overlay to gel at room temperature.

6. Incubate plates at 37^0C in a 5% CO_2 incubator, approximately 7-10 days.

7. Beginning on day 4, examine monolayers in control wells microscopically for plaque formation daily.

8. When plaque formation is well defined and there are an adequate number of plaques in the control wells (40 plaques minimum), fix plates in 10% formalin in PBS. Carefully flip off the agarose layer using a small weighing spatula. Stain the plates with 0.8% crystal violet in 50% ETOH or with methylene blue.

9. Count plaques using an inverted, dissecting, or phase microscope at low power.

VI. RESULTS

A. Calculations

1. Determine mean plaque count for control wells without drug and for each drug concentration.

2. The mean plaque count in control wells with no drug is considered the baseline (100%).

3. For each drug concentration, determine % of plaques remaining compared with control wells without drug.

4. Plot % plaques remaining against drug concentration using semi-log paper.

5. Determine 50% inhibitory concentration (IC_{50}) and 90% inhibitory concentration (IC_{90}).

B. Interpretation

Interpretation of plaque reduction assay results:

Drug	IC50	Interpretation
GCV	$\leq$ 6µM	Sensitive
	>12 µM	Resistant
PFA	< 400 µM	Sensitive

VII. QUALITY CONTROL

To reduce variability, the definition of a plaque should be consistent and one reader should read all the plates.

Note: The area of quality control needs to be further defined in the future. For assay to be acceptable, the following parameters need to be defined:

IC_{50} for control sensitive and resistant viruses must fall within a predetermined acceptable range for that virus strain.

Range of acceptable number of plaques for challenge dose (40-100)

Degree of acceptable secondary plaque formation

Degree of variability in plaque counts between replicate wells

Monolayer condition and degree of monolayer loss

VIII. REFERENCES

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